

AD \_\_\_\_\_

Award Number: W81XMH-08-1-0462

TITLE: The Role of Tumor Microenvironment on Prostate Cancer  
Progression

PRINCIPAL INVESTIGATOR: Clayton Yates

CONTRACTING ORGANIZATION: Tuskegee University  
Tuskegee, Al 36088

REPORT DATE: 2008-12-01

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

x Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. <b>PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.</b>					
1. REPORT DATE (DD-MM-YYYY) 01-08-2009		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 7/15/08 -7/14/09	
4. TITLE AND SUBTITLE  The role of Tumor Microenvironment on Prostate Cancer Progression				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XMH-08-1-0462	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S)  Clayton Yates				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)  Tuskegee University  Tuskegee, AL 36088				8. PERFORMING ORGANIZATION REPORT	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)  U.S Army Medical Research and Materiel Command Fort Detrick, Marvland 21702-				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT  Approved for public release; distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT  Abstract on next page.					
15. SUBJECT TERMS EGFR, Kaiso localization					
16. SECURITY CLASSIFICATION OF: U			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES  14	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

**BACKGROUND:** Current therapies for invasive and metastatic prostate cancer are not curative and prolong survival by nearly a year even in patients with a metastatic disease. Metastasis is a multi-step process wherein tumor cells acquire properties that enable them to detach, migrate, gain access to conduits, and disseminate throughout the body. The dissemination of cancer from the primary carcinoma mass requires a loosening of the cell-cell bonds. Previous investigations have demonstrated that the epidermal growth factor receptor (EGFR) promotes tumor cell invasiveness and metastasis. However, the regulatory control of metastasis genes have not explored the key molecular events mediated by the EGFR induced responses. Recently, Kaiso a biomodal transcription factor, has been shown to be associated with, many cancer-related genes which function as tumor suppressor, such as cell cycle regulator CDKN2A (P16) , TIMPS and E-cadherin. However, a direct link between Kaiso and metastasis as not been shown. Therefore, we hypothesized that Kaiso fits into the EGFR signaling cascade, and is associated with EGFR induced cell migration and metastasis.

**RESULTS:** Prostate cell lines of increasing aggressiveness expressed a trend of cytoplasmic Kaiso expression in less invasive to nuclear in more invasive. To determine the cytoplasmic to nuclear translocalization of Kaiso we utilized DU-145 cells as our model. Initially we treated -DU-145 cell with 10-100ng/EGF and probed for Kaiso expression level by qRT-PCR and immunoblot (Figure 2 a,b). 10ng/ml was found to be the optimal concentration and was utilized throughout remainder of experiments. To determine the specific role of EGFR on Kaiso expression with treated DU-145 cells with EGFR specific kinase inhibitor PD153035. PD153035 completely inhibited the EGF induced increases in Kaiso expression (Figure 2c). 10ng/EGF also resulted in a cytoplasmic to nuclear translocalization of Kaiso as shown by immunofluorescence and immunoblot (Figure 3a,b). As p120ctn is the only known binding partner for Kaiso, we were able to determine that EGF stimulated Kaiso nuclear localization was dependent on p120ctn (Figure 4c), with EGFR activating p120 y96 and y228 specifically (Figure 4a). Furthermore EGF stimulation resulted in Kaiso co-immunoprecipating with p120ctn transiently (Figure 4b). Next, we were able to determine that siRNA Kaiso treated resulted in reexpression of E-cadherin (Figure 5b), and this was associated with a significant decrease cell migration in presence or absence of EGF treatment (Figure 6).

**CONCLUSION:** This data suggests that Kaiso is associated with EGFR signaling cascade via interaction with p120ctn. Furthermore decreased Kaiso levels results in increased E-cadherin expression and decreased cell migration. Our results for the first time show that Kaiso is involved in the molecular machinery regulating prostate cancer metastasis and progression.

## Table of Contents

	<u>Page</u>
Introduction.....	6
Body.....	6-11
Key Research Accomplishments.....	12
Reportable Outcomes.....	12
Conclusion.....	13
References.....	14

## **INTRODUCTION**

The DoD funded Prostate Cancer New Investigator Award" (PC073977) entitled "The role of Tumor Microenvironment on Prostate Cancer Progression. I am pleased to report that these 12 months have been productive and we have been able to accomplish several tasks proposed in specific aim 1 and 2 for this period.

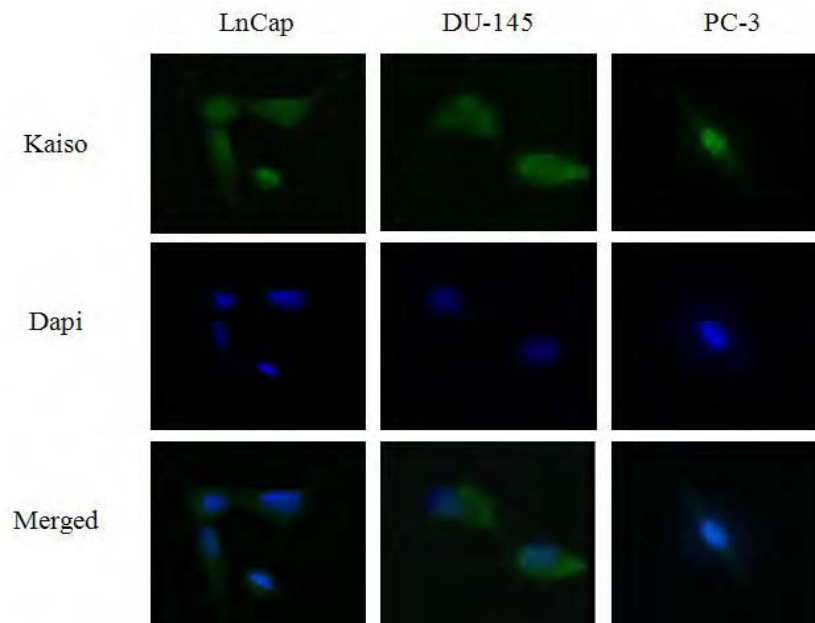
## **TOPIC ADDRESSED DURING THE GRANT**

This proposal will test the hypothesis that Kaiso is a mediator of signals from the cell surface to the nucleus to influence specific gene function, and that it is highly influenced by the tumor microenvironment.

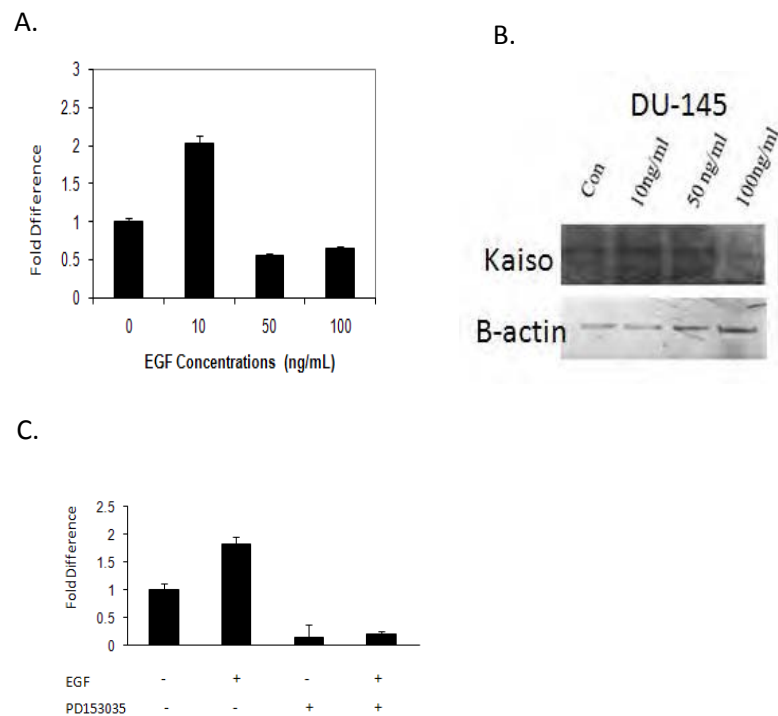
Critical to our model of a reversion of tumor-associated EMT based on the tumor microenvironment is how E-cadherin expression is lost. Intriguingly, and in contrast to the loss of most other tumor suppressors, few sporadic tumors demonstrate mutations or deletions of the E-cadherin gene. Rather, DNA methylation, which is reversible, appears to be the main mode of E-cadherin down-regulation; in fact DNA methylation patterns are unstable in tumor cell lines. In fact, many cancer-related genes which function as tumor suppressor, such as cell cycle regulator CDKN2A (P16) and TIMPS, that has been shown by us and other to play a role in the epithelial to mesenchymal transition in breast and prostate cancers are hypermethylated during aggressive cancer. However, the mechanism through which epigenetic silencing occurs, as consequence of aggressiveness is not well understood.

## **BODY**

Cancer afflicts African Americans to the greatest degree, where African American have a 60% increased risk of developing prostate cancer, twice the risk of developing distant disease, and greater than twice the mortality relative to white men. This trend is also shared in African American women, where breast cancer is diagnosed at more advanced stages, and these women suffer disproportionately compared to their white counterparts. Cancer metastasis is a multi-step processes wherein tumor cells acquire properties that enable them to detach, migrate, gain access to the circulatory system or lymphatic system, and disseminate throughout the body. The ability of the cancer cells to disseminate from the primary carcinoma mass requires a loosening of the cell-cell contacts. A hallmark of this process is the loss of tumor suppressor and cell adhesion molecule, E-cadherin. It has been further perceived that once loss of E-cadherin mediated cell adhesion is acquired, and this is maintained throughout metastasis. Recently, we have observed a reversal of these metastatic properties of the cancer cell by culturing prostate and breast cancer cells with normal cells from the target metastatic organ. We have termed this process Mesenchymal Epithelial reverting Transition (MErT). This results in re-expression of E-cadherin and a transformation of the cancer cell to appear more like a normal cell. These findings have been confirmed in cancer patients that have prostate and breast cancer metastasis to the liver {Yates, 2007 #537; Yates, 2007 #538} and bone microenvironments ({Shah, 2004 #332}. *This led us to hypothesize that epigenetic regulation of gene expression occurs throughout prostate progression, which enables cancer cells to alter their morphology and expression profile to adapt to different microenvironments.* As such we are concentrating on a novel transcription factor, Kaiso, as a mediator of signals from the cell surface to the nucleus to control epigenetically regulated metastasis genes. This proposal explores the role of EGFR signaling on Kaiso during prostate cancer metastasis.



**Figure 1:** Immunofluorescence of Kaiso localization in Human Prostate Cancer cell lines, LnCap (not metastatic), DU-145 (moderately metastatic), PC-3 (highly metastatic) .



**Figure 2** EGF Treatment of DU-145 Cancer Cells. Real-time levels of Kaiso mRNA was analyzed performed with Kaiso specific Taqman primers with HPRT1 as the loading control.(A) and (B) DU-145 cells were treated with 10ng/mL, 50ng/mL, or 100ng/mL of EGF, incubated for 6 hours and probe for mRNA Kaiso levels and protein. (C) DU-145 cells were treated with 10ng/mL of EGF or EGFR specific kinase inhibitor and Kaiso mRNA levels was analyzed by real-time PCR.

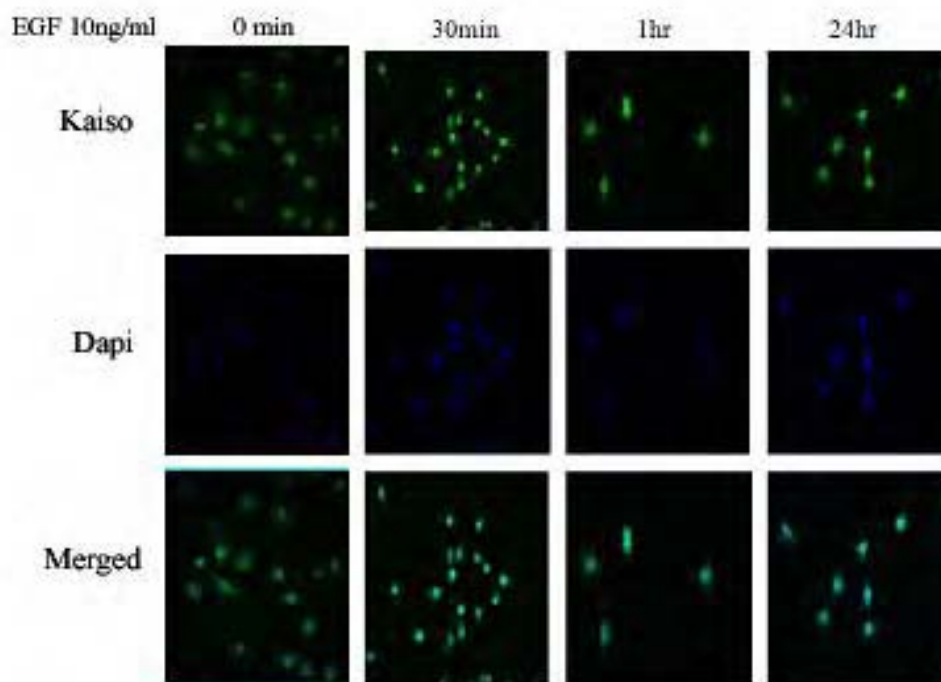
## STATEMENT OF WORK (SOW 12-18 Months)

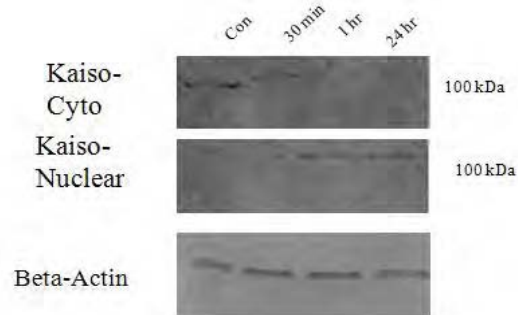
### ***Specific Aim 1. Determine the specificity of EGFR signaling on p120-Y228 mediated membrane to cytoplasm localization of Kaiso.***

To complete the goals this propose, we first performed experiments detailing signaling pathway associated with Kaiso localization (Figure 1). We probed for Kaiso localization in commonly utilized LnCaP, DU-145, and PC-3 prostate cell lines. We have chosen to pursue remaining experiments in DU-145 cell lines has this proposal focuses on the role of EGFR signaling on the cytoplasmic to nuclear localization, within the tumor microenvironment. Our experimental results are detailed below. Materials and Methods employed are provided in the Appendix Section.

#### **1. Activation of EGFR signaling results increased Kaiso expression levels in DU-145 cells.**

Numerous reports have implicated a role for EGFR signaling in prostate cancer progression {Pal, 2005 #431}. Therefore we sought to determine if EGFR signaling would have an effect on Kaiso localization and/or expression in prostate cancer cells. As an initial experiment we performed a dose dependent treatment of EGF consisting of 10ng/ml, 50ng/ml and 100ng/ml. We were able to observe at least a two fold increases in Kaiso RNA and protein levels at only the 10ng/ml utilizing real-time RT-PCR and immunoblot, respectively (Figure 2A). Therefore we utilized 10ng/ml for the remainder of experiments. Next, we sought to determine the specificity of EGFR tyrosine kinase activity on Kaiso expression levels, utilizing EGFR specific kinase inhibitor PD153035 in the presence or absence of EGF treatment. Our results show that 500nM of PD153035 significantly reduced Kaiso expression levels, and PD153035 pre-treatment completely blocked our observed EGF induced increases in Kaiso levels (Figure 2C). Thus, activation of the EGFR signaling cascade has a direct affect on Kaiso expression.





**Figure 3** Activation of EGFR results in cytoplasmic to nuclear localization of Kaiso. DU-145 cells were treated with 10ng/ml of EGF for increasing time intervals. Anti-Kaiso 6F8 was utilized as primary antibody. Anti-Mouse Alexa 488 was utilized as secondary antibody. Upon EGFR activation of Kaiso caused cytosolic to nuclear localization (A). (B) Western blot analysis of WT DU-145 upon treatment of 10ng/ml of EGF.

**2. EGFR results in cytoplasmic to nuclear localization in DU-145 cells.** Our next step was to determine the effect of EGFR increases in Kaiso levels on cytoplasmic to nuclear localization within DU-145 cells. DU-145 cells were treated with 10ng/ml of EGF for increasing time interval of 30min, 1hr, and 24 hours. DU-145 cells were stained with anti-Kaiso and visualized with anti-Alexa 488 by immunofluorescence. Cytoplasmic Kaiso was observed in the DU-145 untreated, however, EGF treatment resulted in Kaiso localization from the cytoplasm to the nucleus as early as 1 hr and this was sustained through the 24hr time period (Figure 3). These findings were further observed upon subcellular fractionation, where 10ng/ml of EGF treatment as early as 30min, resulted in a Kaiso nuclear localization shift. This trend was continued throughout to 24hrs (Figure 3b).



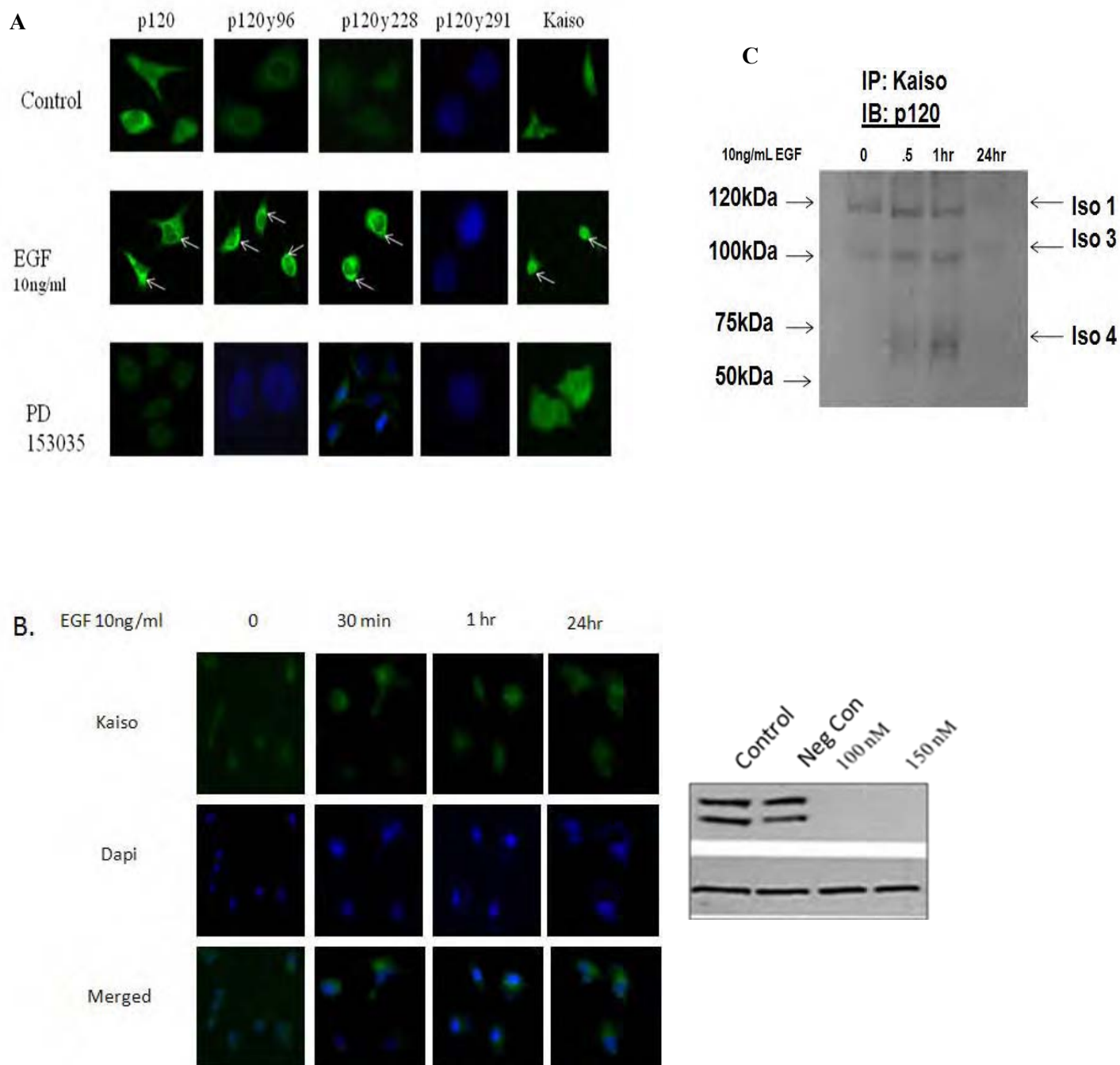


Figure 4. : Immunofluorescence of DU-145 (A). DU-145 cells were treated for 24 hrs with 10ng/ml or PD153035 and probed for p120 Y96, Y288, and Y291 and Kaiso. Secondary antibody Alexa 488 was utilized for visualization(B,) siRNA p120 pretreated cells were subsequently treated with 10ng/ml of EGF treatment for 24hrs.(C). EGF treatment for .5, 1hr, and 24hrs were immunoprecipitated with Kaiso and probed with anti-p120 by immunoblot

### 3. p120ctn is a mediator of EGFR induced cytoplasmic to nuclear localization of Kaiso.

Previous reports have observed that p120ctn has a specific kinase activity for EGFR on tyrosine residue Y228 in squamous carcinoma (Piedra, 2003 #443}. Therefore, we sought to determine whether this was apparent in prostate cancer cell lines as well. Given that p120ctn has multiple tyrosine residues including y228, y96, y291 contained within the regulator domain, we next determined the effect of EGF treatment on these residues. EGF induced expression of Y228 and Y96 expression levels, but not Y291 expression (Figure) after 24 hours. Further highlighting the reversible effects of EGFR signaling on the p120ctn residues, PD153035 was able to decrease Y228 and Y96 expression levels (Figure 4a).

Next, we determined if EGF induced activation of p120ctn was directly responsible for Kaiso cytoplasmic to nuclear localization. Therefore, we immunoprecipitated Kaiso, and probed for full-length p120ctn. Our results, show that EGF induced a p120ctn-Kaiso protein-protein interaction as early as 30min however this interaction was lost after 24hrs of treatment. This interaction appeared to be specific to p120ctn degradation fragments at the 100kda and 60kda, which correspond to p120ctn isoforms 1 and 3, respectively.

To further highlight a role for p120ctn on EGFR induced Kaiso localization, utilized siRNA p120ctn in presence of EGF over 24 hours. In the absence of p120ctn, EGF failed to induced nuclear localization of Kaiso, further highlighting a role for p120ctn during Kaiso nuclear localization.

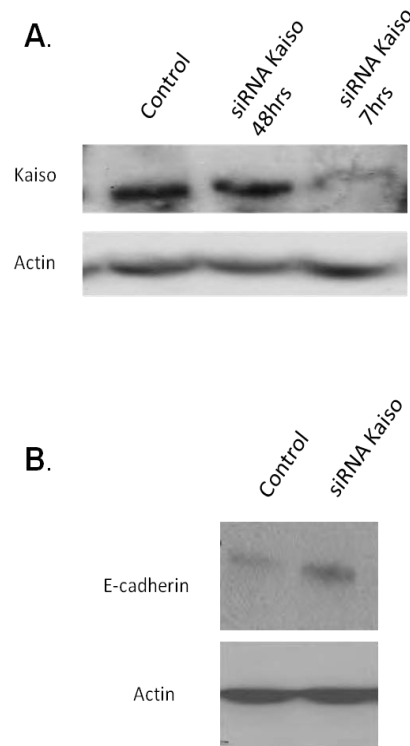


Figure 5 Kaiso controls E-cadherin expression levels. (A) DU-145 cells were treated with siRNA Kaiso for 48 and 72hrs and probed for Kaiso protein levels by immunoblot. (B) siRNA Kaiso treated cells were probed for E-cadherin expression with anti-E-cadherin antibody. Actin served as loading control

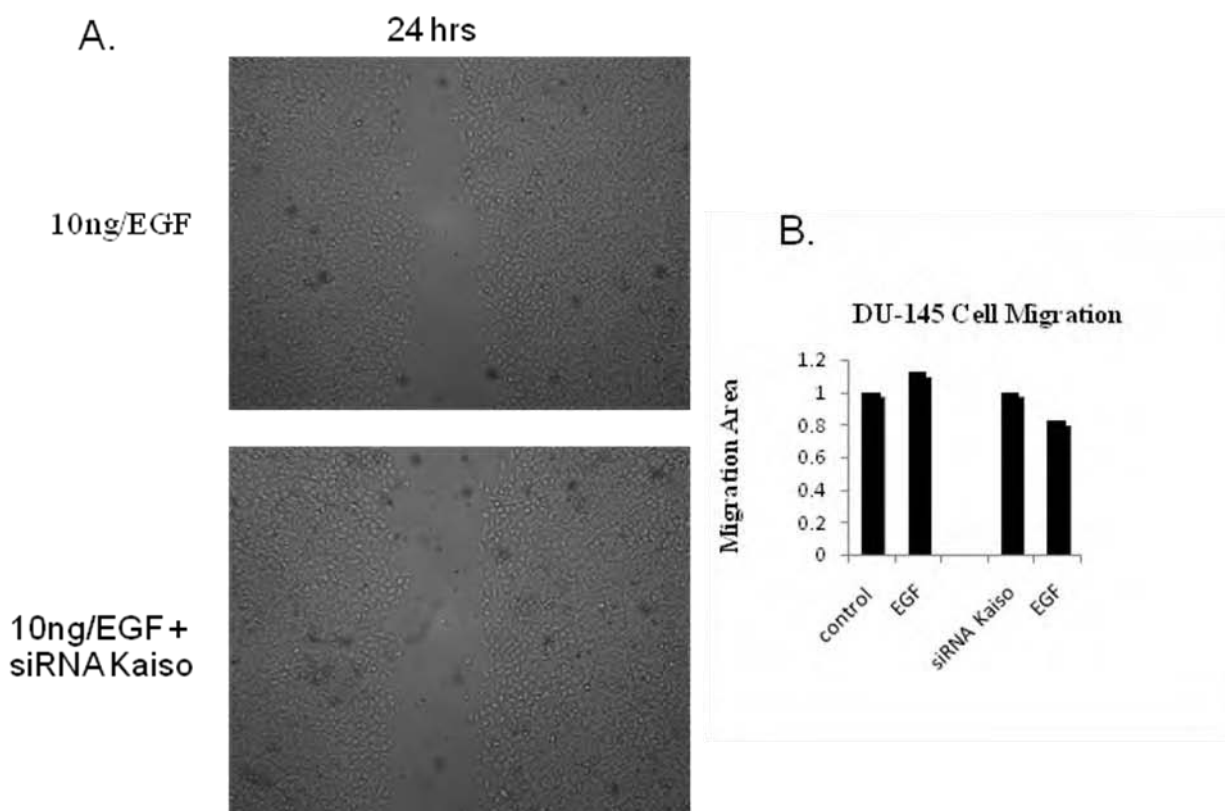


Figure 6 Cell motility in response to EGF. DU-145 cells were treated with EGF. In a two-dimensional in vitro “wound healing” assay DU-145 cells were treated with 10ng/ml of EGF in siRNA Kaiso pretreated cells. (A) is light microscopy of denuded area. (B) Area migrated into wounded area

#### 4. Kaiso is responsible for reexpression of E-cadherin and decreased cell migration.

**(Specific Aim 2c)** Previous findings from our group, highlighted that EGFR signaling negatively effects E-cadherin levels (Yates 2005). Furthermore, Kaiso has been shown to bind to methyl-CpG-specific complex on a fragment of the E-cadherin promoter (Prokhortchouk et al. 2001). Therefore we sought to determine if Kaiso contributes to E-cadherin expression in DU-145 cells. As an initial experiment, we treated DU-145 cells with siRNA Kaiso for 24-48hrs. Significant knock down was observed after 48hrs. Therefore, siRNA Kaiso treated cells after 48hrs were lysed and probed for E-cadherin expression by immunoblot. Our results reveal siRNA Kaiso treated cells showed increased E-cadherin expression (Figure 5b). However, we were not able to observe a difference in the methylation pattern of E-cadherin (data not shown)

Since E-cadherin is a well established epithelial marker, and repression is associated with decrease cell migration and invasiveness of multiple cancers (Ackland, 2003 #3}. We next sought to determine the effect of Kaiso on EGF induced cell migration utilizing cell migration assay. Our results show that siRNA Kaiso did not change the basal migratory (Figure 6 a)

pattern of DU-145 cells, however depleted Kaiso expression significantly inhibited EGF induced area of migration into the denuded area over 24hr time period.

### **KEY RESEARCH ACCOMPLISHMENTS:**

We have achieved significant progress on this project and based upon our data so far, our key research accomplishments are:

1. EGFR induced increased in Kaiso levels and nuclear localization in DU-145 prostate cell lines.
2. EGFR activates p120ctn and subsequently tyrosine residues Y96 and Y228. Activation of p120ctn is required for nuclear Kaiso localization.
3. Depletion of Kaiso with siRNA, is sufficient to reexpress E-cadherin,
4. Depletion of Kaiso is associated with abrogation of EGF stimulated cell migration in DU-145 cells.

### **REPORTABLE OUTCOMES:**

**Data has not been presented in it's entirety. However, several key highlights have been presented at the following national meetings.**

1. 5th Annual National Symposium on Prostate Cancer at Clark Atlanta University, March 16-17, 2009
2. RCMI Health Disparities Symposium, Waik Hawaii –Dec 2008

### **CONCLUSION:**

**CONCLUSION:** In conclusion, this data suggests that Kaiso is associated with EGFR signaling cascade and this is dependent on p120ctn. Furthermore decreased Kaiso levels, results in increased E-cadherin expression and decreased cell migration. Our results for the first time show that Kaiso is involved in the molecular machinery regulating prostate cancer metastasis and progression.

## REFERENCES

- Ackland, M. L., D. F. Newgreen, et al. (2003). "Epidermal growth factor-induced epithelio-mesenchymal transition in human breast carcinoma cells." Lab Invest **83**(3): 435-48.
- Yates, C. C., C. R. Shepard, et al. (2007). "Co-culturing human prostate carcinoma cells with hepatocytes leads to increased expression of E-cadherin." Br J Cancer **96**(8): 1246-52.
- Shah, R. B., R. Mehra, et al. (2004). "Androgen-independent prostate cancer is a heterogeneous group of diseases: lessons from a rapid autopsy program." Cancer Res **64**(24): 9209-16.
- Piedra, J., S. Miravet, et al. (2003). "p120 Catenin-associated Fer and Fyn tyrosine kinases regulate beta-catenin Tyr-142 phosphorylation and beta-catenin-alpha-catenin Interaction." Mol Cell Biol **23**(7): 2287-97.

## MATERIALS AND METHODS

**Cell Culture.** DU-145 cells were grown in high glucose (4.5 gm/l) Dulbecco's modified Eagles medium. This medium was supplemented with fetal bovine serum (FBS; 10%), penicillin-streptomycin (10,000 IU/ml and 10,000 µg/ml), sodium pyruvate (100mM), non-essential amino acids (1X), and L-glutamine (200 mM) (37° C, 90% humidity, 5% CO<sub>2</sub> and 95% air). WT-DU-145 transduced sublines were passaged in G418 (1000mg/ml) until subpassage for experimental testing. LnCap, PC-3, cells were grown in T-media. This media was supplemented with fetal bovine serum (10%) and penicillin-streptomycin (10,000 IU/ml and 10,000 µg/ml) (37° C, 90% humidity, 5% CO<sub>2</sub> and 95% air).

**Immunofluorescence .** Cells were plated on a 4 chamber slide with 20000 cells. After treatment they were then washed with PBS, fixed for 30mins with cold methanol at room temperature, incubated at room temperature with Lysis Buffer containing 0.1 M Tris, 0.15M NaCl, 0.01M EGTA, 1% Triton and 1 mM PMSF, blocked for 30mins in 5% BSA dissolved in PBS, treated with primary antibody (1:100) overnight in the cold room. Cells were stained for p120ctn and Kaiso

**Immunoblotting.** Total cell extracts were boiled for 5 min in Laemmli sample buffer, resolved in SDS polyacrylamide gels, and electrophoretically transferred to PVDF membrane (Millipore, Billerica, MA). For Western blot analysis, filters were incubated in Tris-buffered saline (TBS) solution containing 0.1% Tween 20 and 5% nonfat milk. Filters were then incubated overnight in the cold room with the appropriate antibodies diluted in a TBS also containing 0.1% Tween 20 and 5% nonfat milk. After extensive washing in TBST solution, filters were incubated with horseradish peroxidase-conjugated anti-mouse (GE Healthcare) . The filters were washed as above and developed using chemiluminescence detection system or AP detection system.

**siRNA Transfections.** Cells were incubated until 60-80% confluent. Prepared the following solutions: A: 6 ul of siRNA (Santa Cruz Biotech) into 100 ul of Opti Medium B: 6 ul of Lipofectamine 2000 Reagent (Invitrogen) into 100ul of Opti Medium. Added solution A to B and let incubate at room temperature for 15-45 minutes. During that time washed cells twice with 2ml of Opti Medium. Once incubation was done added 800ul to make a total of 1 ml and then treated cells for 24 hrs. Once treated with 24 hrs let cells recover in normal growth conditions.

**Antibodies.** Monoclonal antibodies to p120ctn, p120ctn y96, p120ctn y228, p120ctn y291, and Beta-actin, E-cadherin, and Kaiso where are commercially available from BD Transduction Laboratories. Immunofluorescent staining for Kaiso was done with Kaiso 6f/6f8 Chip Grade monoclonal antibody from Abcam. Western for Kaiso was done with Kaiso 12H monoclonal antibody from Santa Cruz Biotech.

**Growth Factors and drugs.** Recombinant human epidermal growth factor (EGF) was obtained From Upstate and was added to serum free media of DMEM and T-media with antibiotics. PD 153035 was obtained from Calbiochem and was used at 500nM in serum free media with antibiotics.

**Cell Migration Assay** Cell migration was assessed by the ability of the cells to move into an acellular area in a two-dimensional wound healing assay. At approximately 70–80% confluence, cells were detached and then replated at  $1.0 \times 10^6$  cells/well in 24-well culture plates in complete growth media Dulbecco's modified Eagle's medium (DMEM) and incubated for 24 hours at 37 °C in 5%CO<sub>2</sub>. Cells were then washed with phosphate buffered saline solution (PBS), and the media were changed to DMEM containing 0.5% dialyzed FBS for 24 hours. A denuded area was generated in the middle of each well with a rubber policeman. The cells were then stimulated with EGF (10 nmol/L) in the presence or absence of siRNA Kaiso and then incubated for 24 hours. Images were taken at 0 and 24 hours, and the relative distance moved into the wounded area at the acellular front was determined. All treatments were normalized to the no treatment, which equals 1.